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Characterization of a novel pestivirus originating from a pronghorn antelope

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Abstract

A unique pestivirus, isolated from a pronghorn antelope (*Antilocopra americana*), was characterized. Serum neutralization studies suggested that this virus was antigenically related to pestiviruses. Genomic characteristics, unique to pestiviruses, indicated that this virus belongs to the *Pestivirus* genus. These characteristics included the organization of the 5′ untranslated region (5′-UTR), the presence and length of a viral N^{pro} coding region, conservation of cysteine residues in N^{pro}, conservation of predicted amino acid sequences flanking the cleavage sites between viral polypeptides N^{pro} and C and between C and E^{rns} and conservation of predicted hydrophobicity plots of N^{pro} protein. While this data indicated the virus belongs to the *Pestivirus* genus, phylogenetic analysis in 5′-UTR, N^{pro} and E2 regions suggested that it is the most divergent of the pestiviruses identified to date. This conclusion was also supported by the amino acid identity in coding regions. The corresponding values were much lower for the comparison of pronghorn pestivirus to other pestivirus genotypes than only between previous recognized genotypes. These results suggest the virus isolated from pronghorn antelope represents a new pestivirus genotype. It also represents the only pestivirus genotype first isolated from New World wildlife.

Keywords: *Pestivirus*; Genotype; Pronghorn antelope; Phylogeny

1. Introduction

The *Pestivirus* genus, of the family Flaviviridae, includes economically important infectious agents that infect cattle, pigs and sheep. Currently four species are recognized within this genus; Bovine viral diarrhea virus type 1 (BVDV-1) and type 2 (BVDV-2) infecting mainly ruminants, Classical swine fever virus (CSFV) infecting pigs and Border disease virus (BDV) of sheep (Van Regenmortel et al., 2000). Pestiviruses are not strictly host specific and infect both domestic and wildlife animals. Pestiviruses and/or pestivirus antibodies have been identified in many wild ruminants including deer, roe deer, buffalo, bison, alpaca, eland, kudu, llama, giraffe, reindeer and moose (Anderson and Rowe, 1998; Avalos-Raminez et al., 2001; Becher et al., 1997, 1999; Belknap et al., 2000; Doyle and Heuschele, 1983; Elazhary et al., 1981; Frolich and Hofmann, 1995; Goyal et al., 2002; Hamblin and Hedger, 1979; Kocan et al., 1986; Loken et al., 1982; Motha and Tham, 1992; Nettleton, 1990; Nettleton et al., 1980; Plowright 1969; Van Campen et al., 2001; Vilcek et al., 2000).

The pestivirus genome consists of a positive-single stranded nonpolyadenylated RNA that is around 12.3 kb long (Becher et al., 1998; Collett et al., 1988; De Moerlooze et al., 1993; Meyers et al., 1989; Moormann et al., 1990; Ridpath and Bolin, 1995, 1997). The genomic organization of all recognized pestiviruses is similar. It consists of a large open reading frame (ORF) encoding a polyprotein of approxi-

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mately 4000 amino acids flanked by 5′ and 3′ untranslated regions (5′-UTR, 3′-UTR). The virus-encoded polyprotein is post-translational cleaved by viral and cellular proteases into 11–12 mature proteins. The order of proteins within the ORF is: N^{pro}-C-E^{rns}-E1-E2-p7-NS2/3-NS4A-NS4B-NS5A-NS5B. The first protein (N^{pro}) is the non-structural autoprotease that is unique to pestiviruses and is not found in other Flaviviruses. The next four proteins in order are structural proteins. C is the capsid protein E^{rns}, E1 and E2 are associated with the viral envelope. The remaining gene products are non-structural virus proteins (Meyers and Thiel, 1996).

Nucleotide variation between pestivirus species is observed throughout the genome. The percentage of nucleotide sequence identity is the highest in 5′-UTR (approximately 73–75%). Approximately 71–78% amino acid identity is observed among entire ORF's of BVDV-1, BVDV-2, CSFV and BDV strains (Ridpath and Bolin, 1997).

Several regions of the viral genome have been used for genetic typing of pestiviruses. At present, the 5′-UTR, N^{pro} and E2 regions are most often used (Becher et al., 1997, 1999, 2003; Paton, 1995; Pellerin et al., 1994; Ridpath et al., 1994; Sullivan et al., 1994; Van Rijn et al., 1997; Vilcek et al., 2001). Results of genetic typing revealed that pestiviruses are grouped into similar phylogenetic groups of the genetic region used for analysis, e.g. in non-coding region, coding non-structural or structural proteins. In addition to the BVDV-1, BVDV-2, CSFV pestivirus genotypes first recognized in domestic animals, genetic typing has led to the discovery of additional putative new genotypes. Recent reclassification of BDV strains led to the identification of BDV-1, BDV-2 and BDV-3 genotypes within BDV species (Becher et al., 2003). A pestivirus isolated from reindeer was typed as a separate genotype (Avalos-Raminez et al., 2001) but when more BDV strains were analysed it was reclassified into BDV-2 genotype (Becher et al., 2003). A pestivirus isolated from chamois was typed as BDV-4 genotype (Arnal et al., 2004). The giraffe genotype represents a giraffe strain isolated in 60's and additional strain isolated in 90's (Becher et al., 1999, 2003; Harasawa et al., 2000; Paton, 1995; Van Rijn et al., 1997).

In this report, we describe the isolation and characterization of a novel pestivirus genotype originating from pronghorn antelope (*Antilocopra americana*). The genetic analysis of the 5′-UTR-N^{pro}-C-E^{rns}-E1-E2-NS2/3 genomic region and phylogenetic analysis of the 5′-UTR, N^{pro} and E2 regions shows this virus to be the most divergent pestivirus isolated to date. Characterization of a new pestivirus genotype should contribute to better understanding of pestiviral evolution.

2. Material and methods

2.1. Cells

Initial virus propagation at the Wyoming State Diagnostic Laboratory (Laramie, WY) was done using bovine embryonic testicle (BeT) and ovine embryonic kidney cells (OeK). Virus was also propagated on bovine turbinate (BT) or fetal lamb kidney cells (FLK) at the National Animal Disease Center (ARS, USDA, Ames, IA) and the Diagnostic Virology Laboratory (National Veterinary Services Laboratory, APHIS, USDA, Ames, IA). Cells were grown in minimal essential media (F15 Eagles' medium, GIBCO, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum. Fetal calf serum was tested and found free of BVD viruses and antibodies to BVD viruses. Cells were also free of adventitious BVDV based on immunohistochemistry and polymerase chain reaction (PCR) tests.

2.2. Virus isolation

The head of an immature pronghorn antelope, found wandering blind, was sent to the Wyoming State Veterinary Laboratory. As part of a routine screen for viruses the third eyelid/nictating membrane was removed and prepared as described previously (Van Campen et al., 2001). Primary cultures of BeT and OeK cells were inoculated with this preparate. Cells were subsequently stained with monoclonal antibody Mab 20.10.6 (obtained from Dr. E.J. Dubovi, Cornell University, Ithaca, NY) by indirect immunofluorescent antibody (IFA) method (Van Campen et al., 1997). The Mab 20.10.6 recognizes an epitope of the non-structural protein encoded by the NS2/3 gene and detects BVDV-1, BVDV-2 and CSFV. The virus isolate was sent to the National Veterinary Services Laboratory for characterization. The isolate was inoculated onto BT cell cultures. The BT first passage material was divided and passed twice in BT cultures or primary FLK cells. At the end of the second passage, the cells were stained by indirect immunofluorescent antibody staining method using polyclonal and monoclonal antibodies prepared against the pestivirus E2 polypeptide.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from infected FLK cells was prepared and RT-PCR was performed as described previously (Ridpath and Bolin, 1998). The first fragment (around 280 bp) was amplified from the pronghorn virus using panpestivirus 324 and 326 primers (Vilcek et al., 1994). The starting coding regions were obtained by combination of the panpestivirus 324, 1400RC primer (ACC AGT TRC ACC AMC CAT, where R = A or G, M = A or C), which is a modification of the 1400R primer published by Becher et al. (1997). Further strategy was based on the synthesis of cDNA using primers P5655R (ATT ATN GGT AGN CCT GAC CA, where N = A or G or C or T) and sequencing of PCR product generated using the primers P4900L (GAY GAG WCN GAR TAY, where Y = C or T, W = A or T) and P5537R. Other parts of the virus genome were amplified and sequenced with nine pronghorn specific primers by walking along the se-

quenced regions. The annealing temperature varied in a range 52–56 °C. A single PCR was performed using 35 cycles, the nested PCR using 25 cycles followed with 30 cycles in the second amplification. The length of fragment varied in a range 280–1500 bp.

2.4. Sequencing and sequence analysis

PCR products were purified using Wizard PCR preps DNA purification System (Promega, USA) and sequenced in both directions using the corresponding primers. Sequencing reactions were done using Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Science Inc., USA) and analyzed on a ABI prism 373 sequencer (Applied Biosystems, Perkin-Elmer, USA). Sequences were proof read using SeqManII program from DNASTAR computer program package (Lasergene, Dnastar Inc., Madison, WI, USA). The alignment of sequences was done using Clustal W program (Thompson et al., 1994). Percentage of nucleotide and amino acid identity was taken from the table generated by MegAlign program of DNASTAR. Quality of phylogentic signal in the nucleotide sequences were evaluated likelihood mapping using the TREE-PUZZLE program (Schmidt et al., 2002). Evolutionary distances were calculated using the program DNADIST, employing the Kimura 2-parameter method (Kimura, 1980). Phylogenetic trees were constructed using more different programs, the NEIGHBOR program based on neighbor-joining method (Saitou and Nei, 1987) from PHYLIP inference computer program package (Felsenstein, 1993), the UPGMA method employing the Higgins-Sharp algorithm (Clustal 4) from the MacDNasis software package (Hitashi Software Engineering, San Bruno, CA, USA) as well as the TREE-PUZZLE program and MEGA 2. Statistical analysis of phylogenetic trees was determined by bootstrap analysis (Felsenstein, 1985) carried out on 1000 replicates using PHILIP programs SEQBOOT and CONSENCE. The tree was drawn using the TREEVIEW program (Page, 1996). The hydrophobicity analysis was carried out using PROTEAN program based on method described by Kyte and Doolittle (1982).

The nucleotide sequence from the pronghorn strain described in this work has been deposited in the GenBank library and assigned accession number AY781152. This sequence contains sequences from a portion of the 5′-UTR, the entire N^{pro},C, E^{rns}, E1, E2 and NS2/3 coding regions.

3. Results

3.1. Propagation and preliminary characterization of virus

The pronghorn virus was detectable for more than one to two passages in FLK cells. The titer of virus was achieved low, approximately 10^4 pfu/ml of cell culture supernatant compared to 10^6–10^7 pfu/ml commonly observed in propagation of other pestiviruses. The cytoplasmic staining pattern was similar to that seen with other pestiviruses.

The virus could be neutralized using polyclonal sera propagated against BVDV-1 and BVDV-2 strains. An ovine polyclonal serum collected after hyperimmunization with the pronghorn isolate had neutralizing activity against BVDV-1, BVDV-2 and BDV-1 strains. Thus, there was cross-neutralization between the pronghorn isolate and other pestiviruses.

3.2. Genetic analysis in 5′-UTR and coding regions

3.2.1. 5′-UTR

The alignment of nucleotide sequences of representative BVDV-1, BVDV-2, CSFV, BDV-1, BDV-2, BDV-4 (sequences for BDV-3 were not available), giraffe and the pronghorn strains (Fig. 1) revealed that the pronghorn sequence maintained several motifs found in the 5′-UTR region of other pestiviruses (Ridpath and Bolin, 1995, 1997, 1998). There were observed two variable regions within the 5′-UTR, which are separated with relatively constant region (Fig. 1, position 111–129, sequence underlined with the interrupted line). Compared to other pestiviral 5′-UTR sequences, the pronghorn isolate sequence showed some unique variations in variable regions. In addition to base changes, the computer alignment revealed a significant gap of five nucleotides, which were not observed in other pestiviruses (Fig. 1, region underlined with the full line). A stretch of 22 nucleotides (TCTGCTGTACATGGCACATGGA, where ATG triplet is start codon of ORF) at the end of 5′-UTR and start of ORF was conserved between the pronghorn virus and all pestiviruses used in this analysis (Fig. 1, shared region). The nucleotide sequence identity reported between the four well-recognized pestivirus species in the 5′-UTR ranges between 73 and 75%. However, the sequence identity between the pronghorn isolate and these pestivirus species, in the same region, is lower ranging between 62.6 and 66.8%.

3.2.2. N^{pro}

The alignment of N^{pro} sequences, which are unique only to *Pestivirus* genus, for representative strains of recognized pestivirus genotypes and the pronghorn virus isolate revealed that the pronghorn N^{pro} is similar as for other pestiviruses 504 nucleotides long and encodes for 168 amino acids. All six cysteine residues were conserved. Despite variations in the predicted amino acid sequence, the predicted hydrophobicity profile of the pronghorn N^{pro} is similar to that of other pestiviruses (data not shown).

3.2.3. Other coding regions

The region around cleavage sites for N^{pro}/C, C/E^{rns}, E^{rns}/E1 and E1/E2 are presented on Fig. 2. It should be point out that these cleavage sites were experimentally confirmed in CSFV Alfort strain. Strictly, the amino acid sequences for new pestivirus genotypes represent the alignment around these cleavage sites which were not experimentally confirmed

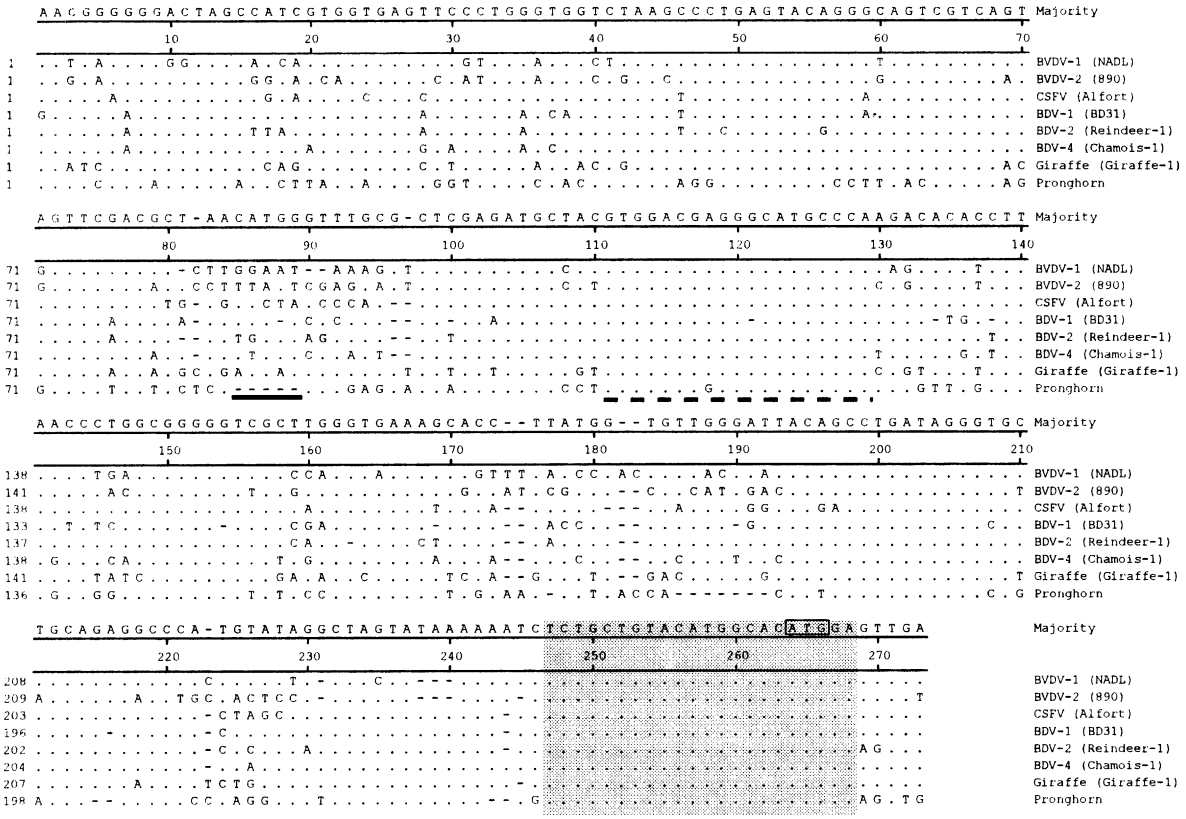

Fig. 1. Alignment of nucleotide sequences in 5′-UTR and at the beginning of ORF. The position of the fragment in the NADL genome is 130–395. The sequences for the representative pestivirus genotypes were taken from the sources as given in Fig. 3. Dots indicate the same sequence as in the consensus line, a dash illustrates a gap. The position underlined with the full line represents five nucleotide long gap in the 5′-UTR of the pronghorn virus genome. The positions underlined with the interrupted line and shaded region represent highly conserved sequences in all pestivirus genotypes. Start ATG codon is boxed.

yet. The most conserved is N^{pro}/C cleavage site both for the C-terminal part N^{pro} and N-terminal part of C protein. The C/E^{rns} cleavage site is more conserved from the E^{rns} part than from the C protein part. The cleavage sites for the E^{rns}/E1 and E1/E2 are quite variable and this variability is the highest for the pronghorn isolate.

Inspection of the alignment in NS2/3 region revealed that there is no cINS, the ubiquitin or other cellular insertions in the pronghorn virus isolate as were observed in some BVDV, BDV and giraffe strains (Avalos-Raminez et al., 2001; Becher et al., 1996; Meyers and Thiel, 1996).

Analysis of the sequence identity can give the first impression on the relationship of the pronghorn isolate to other pestiviruses. When compared the pronghorn virus sequences to BVDV-1, BVDV-2, CSFV, BDV-1, BDV-2, BDV-3, BDV-4 and giraffe pestivirus genotype the amino acid identity varied in the range 55.4–61.3% in N^{pro}, 60.8–70.1% in C region, 50.0–60.4% in E^{rns} region, 45.1–49.7% in E1 region and 39.2–43.0% in E2 region, respectively (Table 1). Thus, the lowest values were observed in E2 region. For comparison, the corresponding values between previous recognized pestivirus genotypes (except the pronghorn isolate) were significantly higher. For example, the amino acid identity between BVDV, BDV, CSFV and giraffe pestivirus genotypes varied in E2 region in the range 52.4–79.9%.

N^{pro}/C ▼	C/E^{rns} ▼	E^{rns}/E1 ▼	E1/E2 ▼	
PLWVTTC SDTKEEG	LFQVTMG ENITQWN	TWFGAYA ASPYCDV	LITGVQG HLDCKEP	BVDV-1(NADL)
PLWVTSC SDEGSKG	LLQLVTG ENITQWN	AWFGAHA ASPYCGV	LITGAQG FPECKEG	BVDV-2 (890)
PLWVTSC SDDGASG	LYQPVAA ENITQWN	TWFGAYA LSPYCNV	LVTGAQG RLACKED	CSFV (Alfort)
PLWVTSC SDDNKNE	MWQPVKP ENITQWN	TWFGANA QSPYCNV	LVTGAKG EFACRED	BDV-1 (BD31)
PLWVTSC SDDNKGE	IPLPVLS ENITQWN	TWFGANA QSPYCNV	LVTGANG QFSCREG	BDV-2 (Reindeer)
PLWLTSC SDEGGAK	LFQPVAG ENITQWN	TWFGAHA ASPYCEV	LVTGAQG AITCEPE	Giraffe(Giraffe-1)
PLWVTSC SDEGGAK	MLHICTA VNITQWN	TWFGAQA SEVYCKV	LLTG-TE ALECDTN	Pronghorn

Fig. 2. Alignment of the amino acid sequences of the representative strains for pestivirus genotypes and pronghorn isolate in the region of the cleavage sites determined for CSFV Alfort strain (Rumenapf et al., 1993; Stark et al., 1993). The arrows indicate the cleavage sites. Position of amino acid in the Alfort ORF: start of N^{pro} position 0, start of C 169, start of E^{rns} 298, start of E1 495, start of E2 690. There were not available complete nucleotide sequence for all structural gene coding regions of the Gifhorn isolate (BDV-3) and chamois pestivirus (BDV-4).

Table 1
Percentage of amino acid identity among the pronghorn virus and different pestivirus genotypes in selected coding regions

N^{pro}	C	E^{rns}	E1	E2	
58.3	60.8	50.0	45.1	41.4	BVDV-1 (NADL)
61.3	60.8	57.7	48.7	40.3	BVDV-2 (890)
59.5	62.9	60.4	46.7	40.9	CSFV (Alfort)
57.1	64.9	59.0	48.7	39.2	BDV-1 (BD31)
61.3	62.9	58.6	49.7	42.2	BDV-2 (Reindeer)
58.3	NA	NA	NA	40.9	BDV-3 (Gifhorn)
57.7	NA	NA	NA	43.0	BDV-4 (Chamois-1)
55.4	70.1	58.6	49.7	40.9	Giraffe (Giraffe-1)

NA: sequences were not available.

3.3. Typing of the pronghorn virus by phylogenetic analysis

The phylogenetic analysis was carried out in the 5′-UTR, entire N^{pro} and E2 regions (Fig. 3), which were the most often used genomic parts for typing of pestiviruses at the genetic level. No significant difference in phylogentic grouping was noted between different programs used. The genetic typing revealed that the pronghorn isolate was placed into a separate phylogenetic branch which is not closely related to the other recognized pestiviral genotypes. The phylogenetic branch with pronghorn isolate was the longest of other branches. Similar position of the pronghorn isolate in the phylogentic trees prepared for three genomic regions indicated that there are not recombinations. The prongorn isolate probably represents a new pestivirus genotype which is the most phylogenetically distant of other pestiviruses identified so far.

4. Discussion

Several lines of evidence support the identification of the pronghorn isolate as a pestivirus. These include: (i) antigenic cross-reactivity with some members of the *Pestivirus* genus; (ii) 5′-UTR motifs; (iii) presence of a putative N^{pro} region located immediately after the 5′-UTR; (iv) conservation of N^{pro} predicted length, cysteine residues and hydrofobicity profile; (v) conservation of the N^{pro}/C and C/E^{rns} cleavage sites.

While the pronghorn isolate may belong in the *Pestivirus* genus there were several observations that suggest it is a new and unique pestivirus species. Unlike other ruminant pestiviruses isolated to date, it was difficult to propagate in cultured ovine and bovine cells. The nucleotide and amino

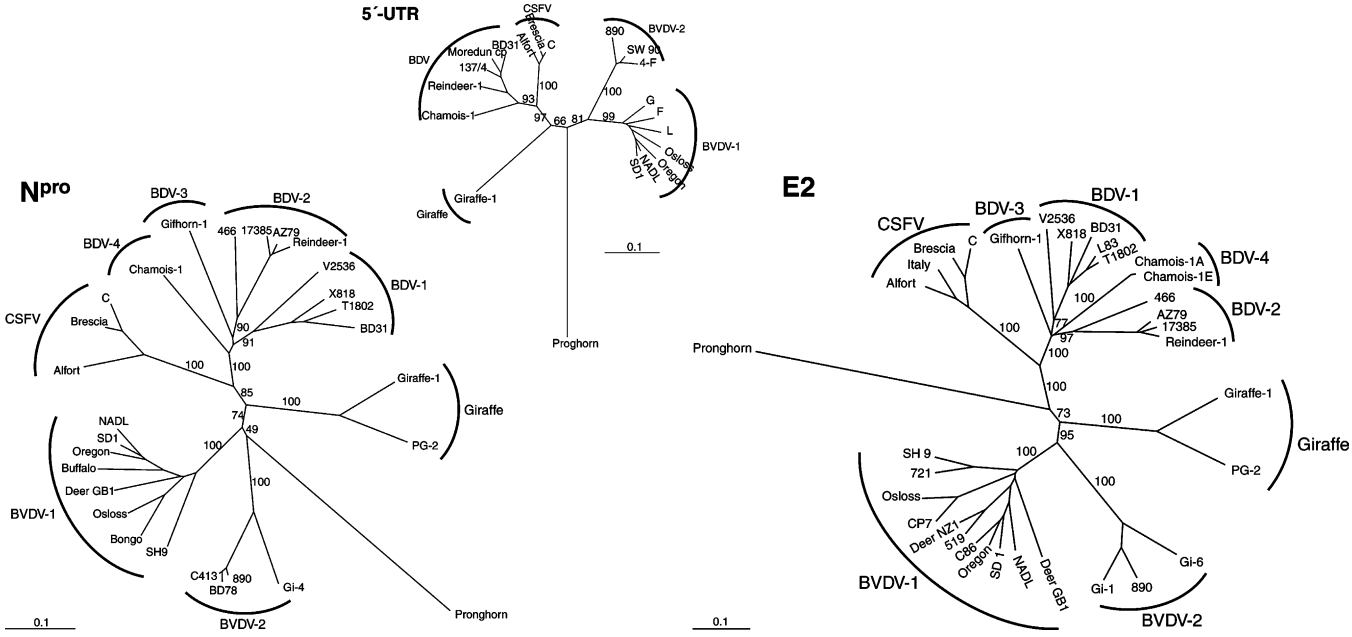

Fig. 3. Phylogenic tree prepared from 245 bp nucleotide sequences of the 5′-UTR (position in NADL: 130–374), the entire N^{pro} (NADL: 386–889) and entire E2 (NADL: 2462–3583) regions. The trees were prepared by neighbor-joining program using Kimura 2-parameter method. Numbers indicate the percentage of 1000 bootstrap replicates that support each group. Sequences for 5′-UTR were obtained from GenBank: NADL (accession no. M31182), Osloss (M96687), SD1 (M96751), 890 (U18059), SW 90 (AB003622), BD31 (U70263), 137/4 (U65052), Mor cp (U65022), Alfort (J04358), Brescia (AF091661), C (Z46258), Giraffe-1 (AF144617), Reindeer (AF144618), Chamois-1 (AY738080). The sequences for strains F, L, G and F4 were obtained from Vilcek et al. (2001). Nucleotide sequences for N^{pro} region were obtained from the same sources as given for the 5′-UTR. Sequences for other strains were taken from GenBank with the following accession numbers: PG-2 AY163647, V2536 AY163648, T1802 AY163649, 466 AY163650, 17385 AY163651, AZ79 AY163652, Gifhorn AY163653, Chamois-1 (AY738083) BD31 U70263, X818 AF037405, DeerGB1 U80902, Buffalo U80901, Bongo AF144474, SH9 AF144473, Gi-4 AF144468, C413 AF002227, BD78 U18330. Nucleotide sequences for E2 region were also obtained from the same sources as given for the 5′-UTR. Sequences for other strains were taken from GenBank with the following accession numbers: PG-2 AY163654, V2536 AY163655, T1802 AY163656, 466 AY163657, 17385 AY163658, AZ79 AY163659, Gifhorn AY163660, Chamois-1A, 1E (AY738081, AY738082), L83 U00890, X818 AF037405, Italy AY027672, 721 AF144609, 519 AF144610, C86 AF144611, DeerNZ1 AF144614, DeerGB1 144615, SH9 AF144616, CP7 AF220247, Gi-1 AF104030, Gi-6 AF144612.

acid identities and especially the phylogenetic analysis of the 5′-UTR, N^{pro} and E2 coding regions demonstrated that this pestivirus isolate is distinct and unique. These results suggest that the pronghorn isolate may represent the first new pestivirus genotype identified in an animal native to the New World.

Identification of a new pestivirus genotype in wild animal raises the question how many pestivirus genotypes exist in nature. Taking into account that four unique pestivirus genotypes (reindeer: BDV-2 genotype; chamois: BDV-4 genotype; giraffe: giraffe genotype; pronghorn antelope: pronghorn genotype) have been identified in wild animals (Arnal et al., 2004; Becher et al., 1999, 2003; this work), and that pestivirus antibodies have been detected in over 40 animal species (Hamblin and Hedger, 1979), it seems logical that more pestiviruses may exist. On the other hand, not all pestiviruses identified in wild animals may represent new pestivirus genotypes. For example, pestivirus from deer (Becher et al., 1997), roe deer (Fisher et al., 1998), mule deer (Van Campen et al., 2001) bongo (Becher et al., 1999), eland (Vilcek et al., 2000), alpaca (Goyal et al., 2002) and buffalo (Becher et al., 1997) were typed as BVDV1, and it is speculated that they were transmitted from domestic cattle. Identification of new pestiviruses may be hindered by the isolation technique and cell culture reagents now in use. Cell lines commonly used for virus isolation may not be adequate to propagate more fastidious or host species specific members of the genus. Reagents currently used for detection may not be able to recognize divergent pestiviruses. The development of a suitable tools and reagents to detect and propagate new pestivirus is a challenge for diagnostic virology laboratories.

At present, it is difficult to suggest the practical significance of our findings. The new pestivirus has only been isolated from one diseased young blind pronghorn antelope. In some parts of the USA, there is close contact between pronghorn antelope and domestic ruminants. Shared rangeland habitat makes transmission theoretically possible. However, there are not experimental data supporting or rejecting such speculation so far. Similarly, the effect of infection on the health of pronghorn antelopes is unknown. The answers to these questions await in vivo studies.

In conclusion, the identification of the pronghorn pestivirus extends our knowledge of the phylogenetic divergence of pestiviruses. Four pestivirus genotypes (BVDV-1, BVDV-2, CSFV and BDV-1) were first isolated from domestic animals and remain economically important pathogens in agriculture production. Recently four additional pestivirus genotypes (BDV-2, BDV-3, BDV-4 and giraffe) were identified. Using this classification, the pronghorn isolate could represent the first member of the ninth pestivirus genotype.

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